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STAIN TECHNOLOGY

VOLUME II JANUARY, 1927 NUMBER 1

PROGRESS IN THE STANDARDIZATION OF STAINS

THE HAEMATOXYLIN PROBLEM

One of the stains which has given most trouble in the matter of standardization is haematoxylin. This is natural, because it is not a synthetic dye and its manufacture cannot be so carefully controlled as in the case of the anilin products. This stain, perhaps, cannot be standardized in any true sense of the term.

Haematoxylin, as is well known, is a glucoside, a constituent of logwood, and the manufacture consists of its removal from commercial logwood extract with ether, followed by crystallization from water.[1] Both of these steps, particularly the first, are slow and difficult to handle on a factory scale, requiring special expensive apparatus. Hence most of the haematoxylin now sold in the United States is the product of a single manufacturer, altho not always sold under the label of this company.

The low solubility of haematoxylin in ether makes the process a slow one, so that even with a relatively large apparatus, the yield per week must be measured in grams, whereas the synthetic dyes may be manufactured in batches of many pounds. A batch from a week's run of haematoyxlin is too small to submit to the Commission for certification; hence the manufacturer has to collect the yield of a long series of runs and mix them all to obtain a composite batch large enough to sample and send in to be tested. This fact presents both advantages and disadvantages. The former arise from the fact that the different runs vary somewhat in color, and a mixed batch is more likely to be of uniform quality than the single runs would be; the latter from the danger of losing the good qualities of some especially fine run thru mixture with others of poor quality. The manufacturers state that the differences in color between different runs, altho the process is not varied from one time to the next, are often large enough to be seen by a glance at the dry product. If the stain were marketed without mixing there would surely be a complaint from users that they could not obtain two lots that were alike.

[1] *See* Houseman, P. H. and Swift, C. K. The preparation of haematoxylin. Indus. and Eng. Chem. **12**, 173. 1920. Continuous extraction apparatus. *Id.* **17**, 830. 1925.

All this makes haematoxylin both difficult to standardize and expensive to manufacture. There have been complaints from users as to the present price; but they do not seem justified. Considering the difficulties (and in fact dangers) of ether extraction on a large scale, the product is bound to be expensive. Comparison of prices seems to show that the imported haematoxylin is now as high in price as the domestic product.

During the war, when the imported supply was first cut off, the haematoxylin supplied in America was, so far as we can find out, a practically unpurified logwood extract. The dark color and rather gummy nature of the material that biologists seem to have obtained at that time certainly suggest that little if any purification had been given it. About the time that this Commission began its activities, however, a product labeled "haematoxylin, c. p." had been put on the market. It was in the form of cream-colored crystals, so light that the product was quite well characterized by the description "white crystals." This purified haematoxylin was found by all to be better than the dark colored domestic product; by some it was claimed to be the best haematoxylin ever put on the market, by others much inferior to the pre-war supply. Such divergence in opinion indicated either prejudice against American stains or great differences in the requirements or technic of the individual users. Criticisms became so insistent that an investigation of the manufacture was made.

The manufacturers were found to be very open in describing their methods and very willing to coöperate. It was learned that so many complaints as to the dark color of the logwood extract had been made that they had resolved to give the users a nearly snow-white product. Accordingly they did the recrystallizing above described in the presence of sulfur dioxide; and the bleaching action of the latter rendered the crystals nearly white. When the matter was taken up with them, they volunteered the suggestion that the sulfur dioxide might have a detrimental effect on the stain. They, therefore, furnished samples for testing, prepared with and without the use of sulfur dioxide.

The samples were tested chiefly in the laboratory of Dr. C. E. McClung at Philadelphia, who subsequently published a brief note on the findings.[2] Results indicated that the haematoxylin was better if not treated with sulfur dioxide, and that it was further improved by a second crystallization. The domestic haematoxylin put on the market during the last three years has nearly all been manufactured

[2]McClung, C. E. Haematoxylin. Science **58**, 515. 1923.

by this method, (i.e. two crystallizations without the use of sulfur dioxide), and has ordinarily been certified by the Commission.

Some of it has not been as good as desired, however. The difficulties in handling the situation have been great. Altho a skilled technician can get a good idea fairly promptly as to whether a sample is a satisfactory stain for certain of the most difficult procedures, that is not the whole story. The chief complaints, in fact, have concerned the keeping quality of the staining solutions, and to test that point naturally requires time. Before such a test could be completed, the manufacturers would have sold out the whole of the batch in question and its approval would do them no good. As a result it was necessary at the start, before they had had time to build up a surplus stock, to compromise by certifying some batches whose keeping-qualities were not fully investigated.

It was not surprising, therefore, that occasional complaints still came in, even of the certified haematoxylin. The matter was again referred to the manufacturers less than a year ago; and it was learned that they had recently made a few slight changes in their process which have had a surprising effect on the product. The new method allows them to accumulate a supply more rapidly and overcomes one of the difficulties of the situation; but unexpectedly it also turns out a more satisfactory haematoxylin. Solutions of it have been kept several months without apparent deterioration. The problem of haematoxylin seems therefore more nearly solved than ever before.

At the present time, samples submitted for certification are tested on sections of actively growing root tips, fixed in Flemming's solution. They are stained by the technic of Haidenhain with an 0.5% solution of haematoxylin. If the sample is satisfactory, the sections should show a sharp, vigorous staining of the chromosomes, without a muddy background. The solution should stain equally well after keeping a month, or longer, if the test can be continued. It is believed that samples meeting these tests will prove satisfactory for all ordinary histological and cytological use.

H. J. Conn

GUSTAV MANN, 1864–1921

LLOYD ARNOLD, *Loyola School of Medicine, Chicago, Ill*

INTRODUCTORY NOTE.—Dr. Gustav Mann is best known to microscopists as the author of the book Physiological Histology. This book (published in 1902) differed from any previous text on histology in the great emphasis it laid on the theoretical side of the subject. A brief study of the book is enough to show that the author was intensely interested in the nature and action of biological stains. Part IV of the book (65 pages) deals with the nature of dyes, the history of staining, and a dicussion of the various staining methods; Part VII (44 pages) discusses the theory of staining; while an appendix (62 pages) takes up in considerable detail the chemistry of dyes, especially those of importance to the biologist. This work was of incalculable assistance in starting the American work on the Standardization of stains. The chapters above mentioned were in fact one of the main sources of inspiration at the outset. The book, Biological Stains, which has been one of the chief accomplishments of this Commission, owed its conception to the suggestion that, Dr. Mann having died, someone else should revise the appendix of his book and bring it up to date. The book, as finally planned, proved radically different from Dr. Mann's treatment of the subject; but we are glad to acknowledge the source of the inspiration.

For these reasons the following brief account of Dr. Mann's life and work, written by one of his former students, should be of interest to the readers of this journal.

—EDITOR.

Doctor Gustav Mann was born in Darjeeling, in the Province of Assam, India, November 6, 1864. His father, a German by birth but a British citizen by adoption, Gustav Adolph Mann, was in the Forestry Department of the British Government service. Doctor Mann's mother died when he was two years old, and as a consequence, his early childhood was spent under the care of a native male nurse. Those of us who knew him could always see the results of this early oriental influence. When six years of age, he was sent to Göttingen, Germany for his education. He lived with, and was practically a member of the household of, a strict but well meaning Lutheran minister. After he had finished the Gymnasium in Göttingen, in 1885, he entered the University of Edinburgh. During these years he had forgotten the little English he could speak when a child, as well as the native language taught by his nurse in India, and could only speak German. He received his M.B. in 1892 and the M.D. in 1894 from Edinburgh. At the age of 25 he won the Ellis Prize as a result of his work upon: "The Respiratory Changes in Fresh Water Plants." Two years before this he was awarded a special prize for his work on "Fertilization of Orchids." These dissertations were followed by a thesis upon "The Evolution of Myosurus." Gradually, under the influence of Professor Rutherford his interest changed from the botanical to the physiological field as is evidenced by his thesis for his M.D. which was upon "The Comparative Study of the Brains of Mammals," and won for him the coveted Goodsir Prize. This was followed by an excellent piece of work upon "The Changes Which the Nervous System Undergoes as the Result of Functional Activity."

Doctor Mann left Professor Rutherford's laboratory in 1894 to become a member of Professor Gotch's staff at Oxford, and remained in this department until he came to America in 1908. He received the B.Sc. from Oxford in 1898. While in the department of Physiology at Oxford he accumulated by literature reviews and by extensive experimental work the material that was embodied in his book "Physiological Histology" (Oxford 1902). As the readers of this journal well know, this work is even at the present time the best treatise upon the chemical reactions involved in the staining of tissue. His conception of histology can best be expressed in his own words, "Histology comprises investigations into the structure, the chemical composition and the function of healthy and diseased animal and vegetable cells, and therefore the ordinarily accepted view that histology concerns itself merely with micro-anatomy seems to me a narrow one."

Following Ehrlich's work upon the use of methylene blue for *intra-vitam* staining, Doctor Mann devoted the most of three years studying the *intra-vitam* technic. He sums up this work in the following sentence in his book; "Much as I should like to deal with the problems of staining and examining living tissues, I feel that as yet I could not have done justice to them, and therefore I have not touched these subjects, other than by giving a full account of the chemistry of the dyes used in the *intra-vitam* technique." Doctor Mann became interested in certain phases of chemistry as a result of the work incorporated in his book on "Physiological Histology." He started to translate Professor Otto Cohnheim's book on "Chemie der Eiweisskörper" in 1904. During the translation he found that there was a gradual accumulation of long foot-notes in which he was offering different views to those expressed in the text by Professor Cohnheim. This led Doctor Mann to publish a book on "Chemistry of the Proteids" (Macmillan 1906) based upon Professor Cohnheim's monograph upon this subject, but differing from it in many respects.

The writer can well remember the first year he was in Doctor Mann's laboratory. This whole year was spent in learning how to *clean* glass ware and *sharpen* microtome knives. I can also remember, at a later time, cutting serial sections, two microns thick, staining the even and odd numbers with different stains and then mounting between coverslips for oil-immersion examination of both sides of the section.

Doctor Mann was Professor of Physiology at Tulane University from 1908-1915. He was in charge of the research chemical laboratories of the Freeport Oil and Fuel Company at Houston, Texas, from

1915 until his death on July 18, 1921. He died in Mexico while pursuing some investigations for this company.

Doctor Mann was married in Edinburgh in 1890 to Miss Agnes Orosz, the daughter of a noted Hungarian revolutionist. He is survived by two sons, one about thirty-five, the other twenty-eight years of age at the present time.

Doctor Gustav Mann was subjected to unusual external environments during early life. As already mentioned, he was left motherless in the Assam Province of India at two years of age. Since his father was absent from home a great deal by the nature of his work in forestry conservation in this province, his early years were spent entirely in the company of his male nurse. This "foster-mother" was a male native of high intelligence who instilled into his young brain an oriental background that Doctor Mann always carried with him throughout his life. He was transplanted from this environment to the home of a Lutheran minister in Göttingen, Germany. From many instances mentioned by Doctor Mann I have gained the impression that this minister was a firm believer in the old adage "to spare the rod spoils the child." The strict and rigid discipline of this environment was one of inhibition to Doctor Mann. His field interests in botany, geology, and zoology during these years were pursued under great difficulties. He passed from this environment at the age of twenty-one into the unrestrained university life of Edinburgh, where he was handicapped by the change from the German to the English language.

Doctor Mann was oriental in his basic reactions; but to an even greater extent, altho somewhat superficially, he was an "echter Deutscher." He was more German to the average acquaintance than English; in fact, he was Teutonic in most of his thoughts and actions, and was only coated with a veneer of British. He was, however, always proud of his British citizenship and liked to think of himself as British. Doctor Mann was a good teacher. He spent a great deal of time preparing his lectures and incorporating into his notes recent literature references. He spoke both English and German with a slight accent. While in England he was a champion debater for the vivisectionist in public debates upon this subject. His "hobby" was collecting Japanese prints. He had one of the best private collections of these prints in America.

Doctor Mann had the calm, quiet and fixed ocular expression of an oriental, an unusual quality in a grey-eyed, broad-featured, Teutonic face. His mustache was always of the ragged English style; in fact, in so far as he was conscious of his environment, he was always

British. In reading any of his publications, the German construction of his long complicated sentences is rather characteristic. This anglo-saxonized Teuton with an oriental-Hindu subconscious background, was loved, honored and respected by those who had the privilege of working under his supervision.

Doctor Mann dedicated his book to Professor Dickson, in Botany at Edinburgh, and part of his dedication reads "to one who aroused my interest in research and taught me patience." As one of his students, I can say that I am indebted to the late Professor Gustav Mann for arousing my interest in investigative work, and teaching me patience.

INVESTIGATION OF THIAZIN DYES AS BIOLOGICAL STAINS

I. The Staining Properties of Thionin and its Derivatives as Compared with Their Chemical Formulae.

Rachel Haynes, *Histologist, Commission on Standardization of Biological Stains, Geneva, N. Y.*

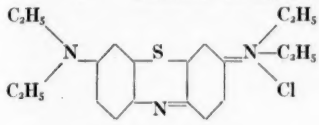
Abstract.—**An investigation has been made of the staining properties of eight dyes of the thionin group. The dyes studied are as follows: tetra-ethyl thionin, asymmetrical di-ethyl thionin, tetra-methyl thionin (methylene blue), tri-methyl thionin (azure B), asymmetrical di-methyl thionin (azure A), symmetrical di-methyl thionin, mono-methyl thionin (azure C), and unsubstituted thionin. The staining properties were tested on sections of paraffin embedded material following five different methods of fixation. No counterstain was employed. It was shown that there was a general correlation between the extent of ethylation or methylation of the dyes and their staining properties. As one passes from tetra-ethyl thionin down the series to thionin itself, there is a progressive decrease in the amount of green showing in the preparations, and an increase in the amount of red present, also an increase in the metachromatic effects, and in the intensity of nuclear staining. There seems, also, to be a similar relation between staining qualities on the one hand and the color and solubility of the dye base on the other.**

Inasmuch as some of the best nuclear stains obtained with anilin dyes are secured with certain members of the quinone-imide group, it was decided to investigate the thionin series. As these dyes differ from one another only in the extent of methylation or ethylation of the amino groups, it was planned to determine if possible, the relation between the number of methyl or ethyl groups and the staining properties. Up to this time, to the author's knowledge, no such investigation has been carried out because of the fact that the pure dyes have not been available. Due to the kindness of Mr. W. C. Holmes of the Color Laboratory of the Department of Agriculture, however, a fairly complete series was obtained for the present investigation.

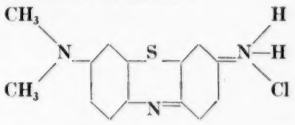
Eight dyes were used as follows: tetra-ethyl thionin, asymmetrical di-ethyl thionin, tetra-methyl thionin (methylene blue), tri-methyl thionin (Azure B), asymmetrical di-methyl thionin (Azure A), symmetrical di-methyl thionin, mono-methyl thionin (Azure C), and unsubstituted thionin. For their chemical formulae, see Table 1. Of these dyes, thionin was a product of the National Aniline and Chemical Company, the methylene blue used was a U. S. P. sample

from the Providence Chemical Laboratories.[1] All of the remaining dyes were laboratory preparations obtained from Mr. Holmes. The Azure B was stated by him to be a crude product, a zinc chloride salt.

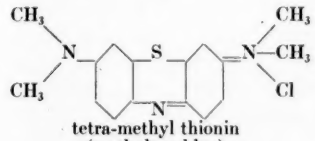
TABLE 1. FORMULAE OF THE DYES INVESTIGATED



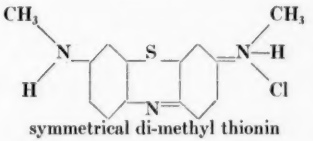
tetra-ethyl thionin



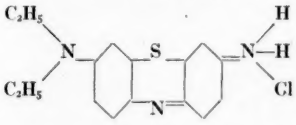
asymmetrical di-methyl thionin (azure A)



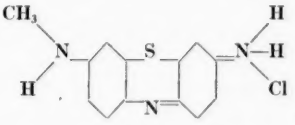
tetra-methyl thionin (methylene blue)



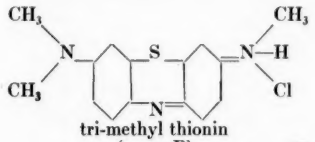
symmetrical di-methyl thionin



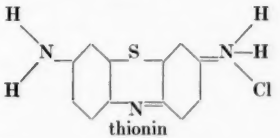
asymmetrical di-ethyl thionin



mono-methyl thionin (azure C)



tri-methyl thionin (azure B)



thionin

TECHNIC

In testing the staining properties of these dyes, four solutions were made up: (1) saturated aqueous solutions, (2) saturated solutions in 95% alcohol plus an equal amount of a saturated solution in anilin oil water, (3) dilute aqueous solutions, (4) dilute aqueous solutions buffered with 0.15 grams of phosphate buffer (reaction pH = 6.5) to 25 cc. of dye solution, These were tested when fresh to observe the effects of the unmodified dyes, and after standing for one or two weeks when certain oxidative changes might have occurred.

All tests were made on sections of paraffin embedded material. Tho the technic and timing were varied, care was taken that all eight slides of each series should be treated in exactly the same manner. Where comparisons were made between one series and another, the

[1]This sample of methylene blue was, as later determined, not pure methylene blue, but contained small amounts of the lower homologs.

material used was always cut from the same block. Thirty-six series of slides were made of material fixed as follows.

Material		*Fixation*
Brain	Mouse	Picro-sulfuric[2]
Brain	Mouse	95% alcohol and 3% bichromate
Brain	Chick	Ohlmacher
Brain	Frog	Zenker
Spleen	Mouse	Bouin
Kidney	Mouse	Bouin
Liver, Pancreas, Digestive System	Fish	Bouin
Liver	Cat	Zenker
Intestine	Frog	Zenker
Ovary	Grasshopper	Zenker

Fresh dilute buffered solutions were tested only on material fixed with picro-sulfuric and Bouin's fluid. Inasmuch as the best results were obtained with these solutions and they were tried out on only two kinds of tissue, the data for them is somewhat incomplete. Series obtained with old dilute buffered solutions, however, were almost as good.

MACROSCOPIC APPEARANCE OF SLIDES

Macroscopic examination of the slides stained with dilute aqueous solution plus phosphate buffer showed that they varied in color from greenish blue to violet in the following order:

Dye	*Number of alkyl groups*
Tetra-ethyl thionin	4 ethyl
(Methylene blue)	4 methyl
Asymmetrical di-ethyl thionin	2 ethyl
Azure B	3 methyl
Symmetrical di-methyl thionin and azure A	2 methyl
Azure C	1 methyl
Thionin	None

The position assigned to methylene blue in this table, altho probably correct, is given to it only tentatively, for no sample of pure methylene blue has been tested.[3] Macroscopic examination of slides

[2]The formulae of these fixing fluids may be found as follows:
Picro-sulfuric. Lee's Microtomist's Vademecum. 8 ed., 1921. See p. 55.
Olmacher. *Id.*, p. 404.
Bouin. *Id.*, p. 62.
Zencker. Mallory and Wright's Pathological Technic. 8 Ed., 1924. See p. 44.

[3]MacNeal's test (see J. Inf. Diseases, **3**, p. 427. 1906) tried out on the methylene blue used resulted as follows: to a strictly fresh dilute solution of the dye was added a small amount of N/10 NaOH and the solution immediately shaken with ether; the ether assumed a red color showing that azure or methylene violet was present in small amounts.

stained with the sample of this dye used in the present work would actually place them nearer azure A in the series.

The effect of the buffered solutions in the above series is of interest. When the experiments were carried out in unbuffered solutions the arrangement of slides by shade of color did not seem to be particularly reasonable: Azure B stained a dull greenish gray; azure C was out of place, for it stained too weakly and was too blue to be placed next to thionin. The fact that these two dyes assumed their theoretically correct positions in the series when used in buffered solutions suggests that these dyes were not of the correct reaction for staining when used in plain aqueous solution.

TABLE 2. SUMMARY OF RESULTS OF A MACROSCOPIC EXAMINATION OF ALL SLIDES.

No. of alkyl groups in dye used:	$4C_2H_5$	$4CH_3$	$2C_2H_5$	$3CH_3$	$2CH_3$ asym.	$2CH_3$ sym.	$1CH_3$	None
Sat. aqu. sol. fresh	*O.K.	Too blue	O.K.	Dull gray	O.K.	O.K.	Very light	O.K.
Sat. aqu. sol. old	O.K.	Too blue	O.K.	Dull gray	O.K.	O.K.	Pale and too green	O.K.
Sat. aqu. sol. old. Slides pretreated with NaOH	O.K.	A little too blue	O.K.	Dull gray	O.K.	O.K.	Light but blue	O.K.
Dilute aqu. sol. fresh	O.K.	Too blue	O.K.	O.K.	O.K.	O.K.	Light but blue	O.K.
Dilute aqu. sol. fresh, buffered to pH = 6.5	O.K.	Too blue	O.K.	O.K.	O.K.	O.K.	O.K.	O.K.
Dilute aqu. sol. old, buffered to pH = 6.5	O.K.	Nearer in place	O.K.	O.K.	O.K.	O.K.	O.K.	M O.K.
Anilin water-alcohol sol. old	O.K.	Too blue	O.K.		O.K.		O.K.	Green blue

*O.K. in the above table signifies that the slide is of such a shade as to fall into its proper place in the color series when the slides are arranged in order according to the molecular weight of the dye used.

A summary of macroscopic examination of all the series of slides made with all solutions may be used to give the results obtained in the most concise form, see Table 2.

It is evident that methylene blue, azure B, and azure C are out of place most often. Thionin is out of position in the anilin-water-alcohol series.

The frequent discrepancy in the case of methylene blue may be explained by the fact mentioned above that it was not a pure sample but contained some azure.

Azure B is also often out of place. When, however, solutions are buffered to a reaction of pH = 6.5, azure B assumes its proper place in the series. Here it is of interest to note that when one series of slides was stained for two weeks in dilute aqueous solutions buffered, azure B stained a dull gray as in unbuffered solutions. When slides, however, were stained for a short time (2 hrs.) in these two week old solutions, azure B slides were again in their proper place in the series.

Azure C is another delinquent; in fresh unbuffered solutions it stains too lightly and in old ones it is also too green. Here again addition of the pH = 6.5 buffer puts it in its place in the series.

Thionin is out of place in only one instance—with the old anilin-water-alcohol solutions where it stains bright greenish blue instead of purple.

As far as has been ascertained, varying the fixative does not make much difference in observing macroscopically the relative color of the sections in these series.

MICROSCOPIC APPEARANCE OF SLIDES

Microscopic examination of the slides placed them in the same general order. A series of slides of mouse brain fixed in picro sulfuric acid and stained in buffered dilute aqueous solutions will serve to illustrate. These slides are used for illustration because several tissue elements are present which differ considerably in staining qualities: cells of various kinds, nuclei, cytoplasm, nerve fibers, and the general matrix. Tetra-ethyl thionin showed scarcely any staining of the nerve cells deeper than that of the rest of the tissue, the whole section appearing an even greenish blue. With methylene blue the matrix was greenish blue, but the cells were stained quite deeply. Azure B showed a slightly less greenish shade, with the cells stained deeper than the matrix, the nuclei especially deep. Asymmetrical di-ethyl thionin was still farther from green in shade and showed slightly deeper stained cells in comparison to the matrix; while azure A and symmetrical di-methyl thionin showed very good cell staining with an apparent increase in purple tones. Azure C was perhaps a bit more purple and the nuclei stood out better than in any of the preceding instances. In the case of thionin the cell nuclei were almost red while the matrix was deeply stained and blue. Another progressive change thruout the series, which this description merely hints at, was the increase in metachromatic effects from tetra-ethyl thionin (where there was no metachromasy) to thionin (where the nuclei stained a reddish purple and the matrix blue). Table 3 will serve to illustrate these points.

This arrangement it will be noticed is in the order of decreasing molecular weight. In the case of the isomers, symmetrical and asymmetrical di-methyl thionin, almost identical staining was obtained.

TABLE 3. RELATION BETWEEN CHEMICAL COMPOSITION AND STAINING PROPERTIES OF THE DYES INVESTIGATED.

No. of alkyl groups in dye molecule	Intensity of nerve cell staining	Amount of green present	Amount of red present	Amount of metachromasy
4 C_2H_5	+	+++++++	+	+
*4 CH_3	++	++++++	++	++
2 C_2H_5	+++	+++++	+++	+++
3 CH_3	++++	++++	++++	++++
2 CH_3 sym.	+++++	+++	+++++	+++++
2 CH_3 asym.	+++++	+++	+++++	+++++
1 CH_3	++++++	++	++++++	++++++
None	+++++++	+	+++++++	+++++++

*This is the theoretical position of methylene blue; the sample used contained some of the lower homologs and should be placed lower in the scale.

PHYSICAL PROPERTIES OF THE DYES AND DYE BASES.

The arrangement also agrees in general with the color absorption of the dyes, the primary absorption band shifting toward the lower wave lengths as one passes over the series in the order named. Formanek's measurements[4] of the absorption spectra of dyes of the series are as follows:

Dye	*Primary absorption maxima in water*	*Secondary absorption maxima in water*
Tetra-ethyl thionin	673.0	614.3
Tetra-methyl thionin	667.5	608.4
Tri-methyl thionin	651.7	596.1
Di-ethyl thionin (asym.)	641.0	590.9
Di-methyl thionin (asym.)	638.0	587.0
Di-methyl thionin (sym.)	620.1	574.5
Mono-methyl thionin	611.4	568.2
Thionin	602.5	559.4

These observations were not obtained by modern methods of spectrophotometry, nor were they made on the same samples of dyes as

[4]Formánek, J. Untersuchung und Nachweis organischer Farbstoffe auf spektroskopischem Wege. Berlin, 1908. Pt. I, p. 153.

used in this experiment. The order of the series agrees, however, with the order of staining obtained with buffered solutions, with the one exception that the positions of azure B and di-ethyl thionin are reversed.

These optical differences are so slight that they are in most cases not noticeable to the unaided eye. An effort was made, therefore, to increase the differences as observed in dilute solutions. This was done by adding to a measured amount of fresh dilute solution of the dyes equal amounts of normal NaOH. The series obtained in this way showed a range of color from greenish blue to red, and when observed directly after making up the solution was as follows:

tetra-ethyl thionin
tetra-methyl thionin
tri-methyl thionin (Azure B)
di-ethyl thionin
mono-methyl thionin (Azure C)
symmetrical and asymmetrical di-methyl thionin
thionin

Azure B was rather dull in color and its position was doubtful. Azure C was not in the position one would expect from its chemical formula. This series agrees exactly, however, with the staining results obtained with dilute unbuffered aqueous solutions. If this same series of solutions is observed after standing a short time, the methylene blue solution stands conspicuously out of place, for it should now be placed much nearer the purple end, altho when the series is first observed it is much greener even than its isomer, asymmetrical di-ethyl thionin. This fact seems at variance with the staining behavior of this sample; but the explanation is probably as follows: When a methylene blue solution is treated with NaOH, the relatively insoluble bases of the azures and methylene violet are precipitated, leaving only the readily soluble methylene blue base to impart a greenish blue color to the solution; as the solution stands, more of the lower homologs are formed and the solution becomes "polychrome methylene blue."

A further test was made by adding to a measured amount of a saturated solution of each dye a constant amount of NaOH, and then a measured amount of distilled water. This treatment should convert the dye salt into the dye base and bring out the color of the latter, with or without precipitation according to the degree of solubility of the dye base in water. With tetra-ethyl thionin no precipitate was formed. Methylene blue showed a slight precipitate. With azure B the precipitate was very slight but more than with methylene blue. An appreciable precipitate was formed in the case of di-ethyl thionin,

altho quite a little blue remained in solution; this was also true of symmetrical di-methyl thionin. Azure A and azure C, on the other hand, formed a gummy looking, brown precipitate and little dye remained in solution. Thionin showed a heavy red brown precipitate and almost no dye left in solution.

The order of solubility of the dye base[5] roughly determined in this manner is as follows:

tetra-ethyl thionin
tetra-methyl thionin
tri-methyl thionin
di-ethyl thionin
di-methyl thionin
mono-methyl thionin
thionin

Since the amount of dye still dissolved in the supernatant fluid was of interest, equal amounts of this fluid were diluted with measured amounts of distilled water and compared. A series resulted going from the deep blue of tetra-ethyl thionin down to the almost colorless solutions of azure C and thionin.

It is interesting to notice that in all these methods of comparison by means of the physical properties of the dyes or their bases, the series obtained is essentially the same as that observed in the case of their behavior as stains.

DISCUSSION.

It is generally known that basic dyes are better nuclear stains than acid dyes, and it might easily be assumed that the stronger bases would be better nuclear stains than the weaker bases. It is apparent in this series, however, that the weaker bases are the best for this purpose. The stronger bases of the series are tetra-ethyl, di-ethyl, and tri-methyl thionin, and methylene blue, because they have the more highly substituted amino groups. With the exception of methylene blue, these dyes prove to be the poorest nuclear stains of the series; and the nuclear staining properties of methylene blue are generally supposed to be due not to this dye itself, but to the lower homologs of the series (i. e. weaker bases) that are usually present, and were found in the sample studied.

It is very evident from the present work that the number of substituent alkyl groups not only correlates with the color of the dye

[5]It is not certain that only the dye base is formed when these solutions are treated with NaOH; in fact, with the methylene blue solution it is certain that the precipitate formed is not methylene blue base. This test actually determines the relative solubility of either the dye base or the bases of the dye or dyes formed upon treatment with NaOH.

(as is well known by the dye chemist), but also with the staining behavior of the dye. As one passes from tetra-ethyl thionin down the series to thionin itself, there is a progressive decrease in the amount of green showing in the preparations, and an increase in the amount of red present, also an increase in the metachromatic effects, and in the intensity of nuclear staining. Altho there were a few exceptions, this correlation holds true in general, and is most evident when the dyes are made up in buffered solutions.

There is also an apparent relation between solubility and color of the dye base on the one hand, and the staining properties of the dye solutions, on the other; for the better stains of this group have the more insoluble bases, and also the greater divergence in color between the dye base and the dye salt. This relation, if it actually holds, is very interesting, especially if it is taken into consideration that proteins are supposed to act as amphoteric electrolytes; and it merits further investigation.

Other points calling for further investigation seem to be: the effect of buffer solutions of different reactions upon these dyes, not only when incorporated in the staining solution, but also when used for pre-treating or post-treating the slides (as suggested by Naylor's work);[6] and comparison of the behavior of these same dyes in double staining, particularly when following some dye of the eosin group.

The author wishes to thank Dr. H. J. Conn for helpful suggestions and ideas in this work and Mr. W. C. Holmes without whose aid in supplying dyes this investigation could not have been carried out.

[6]Naylor, E. E. 1926. The hydrogen-ion concentration and the staining of sections of plant tissues. Am. J. Bot. **8**, 263–275.

THE PREPARATION OF NEUTRAL RED IODIDE SUITABLE FOR VITAL STAINING[1]

MAX PHILLIPS, *Associate Chemist*, and BARNETT COHEN, *Chemist*, *Hygienic Laboratory, United States Public Health Service*

In connection with a certain investigation now in progress in this laboratory, it became necessary to prepare pure *neutral red*. After several trials it was found by somewhat modifying the method first proposed by Witt[2] that neutral red iodide could be obtained, which upon analysis gave results agreeing closely with that calculated from its generally accepted formula (Colour Index No. 825).

Various investigators have observed that post-war neutral red (including Grübler's) produces atypical vital staining and possesses toxicity, disadvantages which were absent in the Grübler pre-war product. It was, therefore, of interest to determine the vital staining properties of our preparation of neutral red iodide. Samples were submitted for testing to two different investigators who have reported that our compound is apparently equal in staining properties to the pre-war neutral red of Grübler.

An authentic sample of pre-war Grübler neutral red, which gave satisfactory results when used for staining, was analyzed and found to be the chloride of the color base and not the iodide. No explanation can be offered at this time for the unfavorable staining qualities of American-made neutral red and the post-war Grübler product, other than that there are apparently certain impurities present in these preparations which in some way interfere with the staining process. It appears that the only advantage of the iodide over the chloride is that it is more readily prepared in a high state of purity.

EXPERIMENTAL

PREPARATION OF TOLUYLENE BLUE (COLOUR INDEX NO. 820)

Thirty-six grams of nitrosodimethylaniline hydrochloride[3] was dissolved in 500 cc. hot water and allowed to cool to 30°C. To this was added, with continuous stirring, a solution of 24 g. m-toluylene diamine dissolved in 500 cc. water and the reaction mixture allowed to stand at room temperature for several hours. The dye separated out in the form of glistening blue crystals. Yield, 80 per cent of theory. A sample of this was dried at 80°C. in partial vacuum (12 mm.) for 24 hours and analyzed for nitrogen by the absolute method.

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[1]Published by permission of the Surgeon General, United States Public Health Service.

[2]Witt, O. N. Ueber neue Farbstoffe. Ber. Deut. Chem. Ges. **12**, 931–939. 1879.

[3]This was prepared according according to the method given in L. Vanino's Präparative Chemie, **2**, 669. 1923 ed.

Sub., 0.1095 g., yielded 17.2 cc. N_2 (763 mm., 25°C.)
Calculated for $C_{15}H_{19}N_4Cl + H_2O$: N, *18.14*
Found: N, *18.07*

Conversion of Toluylene Blue into Neutral Red Iodide

Twenty-five grams of toluylene blue was dissolved in 500 cc. water and boiled for one-half hour while at the same time a current of air was passed through the solution. To this was added 25 grams stannous chloride dissolved in the minimum amount of water. On standing, crystals of the stannous chloride double salt of neutral red separated out, which were filtered off. This was dissolved in water and a strong aqueous potassium iodide solution was added. A dark-brown precipitate of the neutral red iodide separated out, which was filtered off, dissolved in water and re-precipitated with potassium iodide solution. This was filtered off, air-dried, and crystallized from boiling 95 per cent ethanol. It was obtained as glistening green needles. M.P. 261° C (corr.).

Analysis: sub., 0.2000 g., AgI, 0.1262
sub., 0.1085, yielded 13.9 cc. N_2 (762 mm., 27°C.)
Calculated for $C_{15}H_{17}N_4I$: I, 33.38; N, 14.73
Found: I, 33.41; N, 14.62

This work is being continued.

THE ADJUSTMENT OF THE CONCENTRATION OF VITAL STAINS BY COLORIMETRIC METHODS.

ALDEN B. DAWSON, *Department of Biology, New York University.*

Recently, through the courtesy of Dr. H. J. Conn, I had the opportunity of testing a sample of purified American neutral red against a sample of pre-war Gruebler neutral red for relative toxicity, and at his request I am making this brief report. Solutions of both dyes, 1 to 5,000, were prepared and tested, using Paramoecium and Euplotes to measure the degree of toxicity. In this test the American sample was found to be markedly more toxic than the Gruebler product.

It was noted, however, that even in the large bottles the solution of American dye was of a deeper color, indicating that the powder had a higher dye content than the Gruebler dry stain. Accordingly, solutions of the dyes were compared colorimetrically, and it was found necessary to dilute the American solution to 1:6666 before colors of the same intensity were secured.

When the corrected solution of American dye was tested against the Gruebler solution of 1:5000 the difference in toxicity was found to have been practically eliminated.

In comparing solutions of dyes which are sensitive to variations in the H-ion concentration it is necessary to keep the H-ion concentration uniform. It may be either neutral or distinctly acid or alkaline, for such a dye as neutral red which is yellow at pH = 6.8 and crimson at pH = 8.0.

From the above experiment it is obvious that the method commonly used by biologists of preparing solutions of vital stains by making a "percentage solution" rather than a normal or molar solution, is open to serious error unless the dye content of the dry stains produced by the various manufacturers is uniform. It is probable that the salt and moisture content are not always the same and often are not known. Such difficulties would be avoided, partially at least, if each manufacturer made a quantitative analysis of each batch of stain and placed this information on the label. Since vital stains are often used in extreme dilution from 1:5000 to 1:500,000, such quantitative variations may be the cause of serious discrepancies in the results of different investigators if they are using stains produced by different manufacturers or different batches of stains made by the same manufacturer. By using a colorimetric method it is possible for a single investigator to make up solutions of more nearly equal dye concentration even if he uses stains obtained from several sources. Such standardization is only relative, however, and has a limited value.

Absolute standardization could only be secured by preparing a standard solution from a carefully prepared stain and then evaluating each batch of stain in terms of this accepted standard solution.

In addition to this difference in dye concentration, the unknown chemicals in an impure dye may modify the rate and degree of penetration of the dye through the cell membrane, and may also alter the toxic properties of the solution.

STABILITY OF THE GRAM REACTION

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ABSTRACT.—**In the course of about 7000 examinations of Gram-positive aerobic bacteria, it was found that two distinct groups of Gram-positive organisms could be recognized. One group was illustrated by *Micrococcus freudenreichii*, the other by *Bacillus anthracis*. All individuals in a smear of the former remained positive even when the time of exposure to stain was greatly diminished and the time of exposure to decolorizer was greatly increased. Similar changes of technic when *B. anthracis* was used showed that about 70% of the individuals in a given smear became Gram-negative. The writer accordingly distinguishes between stable and unstable Gram-positive bacteria.**

During the course of a study of the effect of certain agents on the Gram reaction of bacteria, about 7000 examinations have been made of many Gram-positive aerobes. The technic suggested by Burke was adopted for these experiments, after trial of various others, because with this method the results are absolutely clear cut, the colors in the final smear contrast sharply and one is practically never left in doubt as to whether an individual organism is to be called Gram-negative or Gram-positive.

In making these studies two smears were stained by two different observers (C. and S.) and each of these smears examined by each observer. Four observations were thus made by two different individuals on each organism. For the great majority of the organisms these observations agreed very closely, but a few strange exceptions were encountered. In these exceptional instances the specimen stained by one observer might contain 95% Gram-positive forms while a specimen of the same organism stained by the other observer might contain only 20% to 40% Gram-positive forms. It seemed probable that this disagreement might be due to very slight variations in the technic used by the two observers. A study was therefore made to see what the effect on different Gram-positive organisms would be of variations in time of exposure to stain, to mordant, and to decolorizer. It was found that for many organisms (*Micrococcus freudenreichii* might be cited as a typical example) variations in time of exposures made, within wide limits, practically no difference in the results, *M. freudenreichii* being always 99% to 100% positive. In the case of other Gram-positive (organisms 99% to 100% positive when the times of exposure advised by Burke were exactly followed),

TABLE 1. RESULTS OF THE GRAM STAIN ON THREE DIFFERENT ORGANISMS.

Organism:	Colon organism				*Bacillus anthracis*				*Micrococcus freudenreichii*				
Observer:*	SS	SC	CC	CS	SS	SC	CC	CS	SS	SC	CC	CS	
					Percentage of positives								
1. Stain—5 sec. Iodine—5 sec. Decolorizer—5 min.	Neg.	Neg.	Neg.	Neg.	35%	40%	20%	35%	100%	98%	100%	100%	
2. Stain—15 sec. Iodine—15 sec. Decolorizer—2 min.	Neg.	Neg.	Neg.	Neg.	80%	80%	60%	50%	100%	100%	100%	100%	
3. Stain—30 sec. Iodine—30 sec. Decolorizer—1 min.	Neg.	Neg.	1%	Neg.	100%	100%	90%	95%	100%	100%	100%	100%	
4. Stain—3 min. Iodine—1 min. Decolorizer—10 sec.	Neg.	Neg.	Neg.	Neg.	100%	100%	99%	100%	100%	100%	100%	100%	Burke technic.
5. Stain—4 min. Iodine—2 min. Decolorizer—5 sec.	Neg.	Neg.	Neg.	Neg.	100%	100%	100%	100%	100%	100%	100%	100%	
6. Stain—10 min. Iodine—5 min. Decolorizer—5 sec.	Neg.	Neg.	Neg.	Neg.	100%	100%	100%	100%	100%	100%	100%	100%	

*Two individuals (S. and C.) took part in these tests. Each made a set of slides and examined both sets. In this table:
SS indicates slides made by S., read by S.
SC indicates slides made by S., read by C.
CC indicates slides made by C., read by C.
CS indicates slides made by C., read by S.

wide variations in result could be obtained by varying the technic. These facts are well shown in the protocol of one experiment. (See Table 1.)

The colon organism used for this experiment was isolated by Torrey from the stool. The *Bacillus anthracis* was obtained from the New York City Board of Health, the *M. freudenreichii* from the American Type Culture collection (No. 40). Nineteen hour cultures of the colon organism and *M. freudenreichii* and six hour cultures of *B. anthracis* were employed for this experiment. Two sets of six slides each were made, every slide containing smears of all three of the organisms. One set was stained by S. and read by S. and by C. (recorded as S.S. and S.C. in the protocol). The other set was stained by C. and read by C. and by S. (recorded as C.C. and C.S.). Six different modifications of the Burke technic were used, varying from one in which the stain and mordant were left on the slide 15 minutes and the decolorizer applied for only 5 seconds (technic 6) to one in which stain and mordant were left on only 5 seconds each and the decolorizer applied for 5 minutes (technic 1). When stain, mordant and decolorizer were used as advised by Burke (technic 4) the colon organism was entirely negative; *B. anthracis* (except for an occasional negative form in one smear) and *M. freudenreichii* were entirely positive. When the time of application of stain and mordant was diminished and the time of application of decolorizer increased, greater and greater numbers of Gram-negative forms began to appear in smears of *Bacillus anthracis*. Although *M. freudenreichii* remained practically 100% positive when technic 1 was used (stain 5 seconds, iodine 5 seconds, decolorizer 5 minutes) nearly 70% of the organisms in the smear of *Bacillus anthracis* were Gram-negative.

It is thus clear that *B. anthracis* and *M. freudenreichii*, tho both Gram-positive organisms, are Gram-positive in a somewhat different sense. The Gram-positivity of *M. freudenreichii* appears to be a stable characteristic, and is not upset by wide variations in staining technic. The Gram-positivity of *B. anthracis* on the other hand is unstable; by shortening the period of application of stain and mordant and prolonging the period of application of the decolorizer, the organisms can be made to appear largely Gram-negative. This distinction between stable and unstable Gram-positives appears to be a useful one. It is suggested that in recording the Gram reaction of new species, some such set of comparative tests as here described be carried out so that the stability of the reaction be known.

DISCUSSION OF PAPER ON "BRILLIANT-GREEN-BILE MEDIA"[1]

COMMENT BY FRANK E. HALE,

Director of Laboratories, Dept. of Water-supply, Gas and Electricity, New York City

Casual reading of the above-named article would lead the reader to feel that a change may be made to the use of the bile media showing maximum growth of the colon organism, 2% bile and 1:75,000 brilliant-green. This *may not be done*, however. The work in question was carried out entirely with the colon organism, and while very important in showing that the original media does not inhibit fresh cultures of this organism, yet no work was done with *Bacillus Welchii*.

Unfortunately the 2% bile and 1:75,000 brilliant-green permits *B. Welchii* to grow vigorously, hence *cannot be substituted* for the original media of 5% bile and 1:10,000 brilliant-green. In June of this year at Mr. Prospect Laboratory tests were made on 2% bile and varying amounts of brilliant-green with *B. Welchii*. Cultures obtained from Washington for some reason failed to produce any gas on testing in plain bile (possibly they were of the type that grow only in broth); consequently a fresh culture of *B. Welchii* was isolated from garden soil. This culture gave 75% of gas in 5% bile with no brilliant-green, but no gas in 5% bile and 1:10,000 brilliant-green. The following table shows the results of duplicate tests in 2% bile and varying amounts of brilliant-green.

Bile	*Brilliant-green.*	*Percent gas.*			
		24 hours.		*48 hours.*	
2%	1:10,000	0	0	0	0
2%	1:25,000	0	0	0	0
2%	1:50,000	B*	B*	5	12
2%	1:75,000	B*	B*	3	3
2%	1:100,000	B*	B*	10	5
2%	None	30	30	30	30

*Decolorized.

It is obvious that not until the brilliant-green reached a strength of 1:25 000 was *B. Welchii* inhibited. Lauter, at Washington, in working with the Committee of the American Water Works Association investigating brilliant-green bile, inadvertently planted 10cc. of water in 10cc. of media (5% bile and 1:10,000 brilliant-green) and obtained practically as good results both in growth of the colon organism and inhibition of *B. Welchii* or spore-formers in general. (Paper read at Buffalo Convention American Water Works Associ-

[1]H. G. Dunham and H. W. Schoenlein. Stain Techn. 1, 129–134. 1926.

ation, 1926.)[2] That means that his final strength of media, as incu bated, mixture of water and media, was 2.5% bile and 1:20,000 brilliant-green. It is interesting to note that the amount of brilliant-green in the above table, in Lauter's work, and in the original media necessary to inhibit spore-formers is exactly inversely proportional:

Bile.	*Brilliant-green.*
2%	1:25,000
2.5%	1:20,000
5%	1:10,000

that is: 10,000:25,000 = 2:5.
10,000:20,000 = 2.5:5
20,000:25,000 = 2:2.5

It would therefore seem that the relative amounts of brilliant-green to bile cannot be changed if we wish to inhibit spore-formers; and that is the purpose of the media. In the three years work of the Committee of the American Water Works Association using 5% bile and 1:10,000 brilliant-green there was only 0.5% error with regard to spore-formers in tests carried out in all sections of the country.

Now as to the colon organism, Table 2 in Dunham and Schoenlein's paper indicates that, even using fresh cultures, 2% bile and 1:20,000 brilliant-green inhibited this organism somewhat so that the counts were only 64 against 100 with no brilliant-green. But table 3 demonstrated that 5% bile and 1:10,000 brilliant-green stimulated the colon organism somewhat so that the counts were 118 against 100 with no brilliant-green. In other words the slight inhibition which brilliant-green exerts on growth of this organism is neutralized by the correct amount of bile.

From all the data so far published as well as extensive research unpublished it seems unwise to change the constituents of the original media from 5% bile and 1:10,000 brilliant-green, considering also the diluting effect of the water examined. The Department of Water Supply Laboratories of New York City using Smith fermentation tubes employ a final strength of bile, when diluted with the water examined, of 4% with corresponding resulting dilution of the brilliant-green. Many other laboratories like Lauter, using Dunham tubes, undoubtedly employ a final dilution of 2.5% bile and a corresponding amount of brilliant-green. Results would seem safe between these limits. The use of the media would be futile if changes were made which permitted spore-formers to grow. *It is far preferable and indeed advantageous to inhibit some of the attenuated colon organisms which have little if any sanitary significance.*

[2]C. J. Lauter. Earlier determinations of Bacterium colon. J. Amer. Water Wks. Assn. 16, p. 625. 1925.

REPLY BY H. G. DUNHAM AND H. W. SCHOENLEIN.

The paper, "Brilliant Green Bile Media" commented on above by F. E. Hale, as he said, considered the colon organism only. The general objection to the brilliant green bile medium with 5 per cent. bile and 1:10,000 brilliant green has been that some forms of this organism were inhibited. It was for the reason of this objection that one section of Standard Methods of Water Analysis, Committee No. 1 of the American Water Works Association, carried on this work. Our problem was to find a combination of bile and brilliant green that would not inhibit the colon organism. Later on it was planned to put such a medium as we could find to a practical trial, and this is being done this year by Committee No. 1 under the direction of Harry E. Jordan. Mr. Jordan is now having two media tried out under practical conditions in laboratories scattered all over the country. Until such time as Mr. Jordan's work is reported, the authors will be unable to comment upon the actual value of the brilliant green bile media under practical conditions.

The question resolves itself into 2 possibilities. Shall we have a medium which inhibits all *Bacillus Welchii* and some colon forms, or a medium which does not inhibit the latter and which might possibly let thru an occasional one of the former? The authors of this paper have shown several combinations of brilliant green and bile that do not inhibit the strains of the colon organism used, and further show that some combinations exert a slight stimulation.

The paper referred to above was a report of laboratory findings of a preliminary nature and is being followed up by Mr. Jordan in a practical way in a large number of laboratories thruout the country to determine whether these media will or will not inhibit non-confirming lactose fermenters.

COMMISSION SPECIFICATIONS OF CERTAIN STAINS.

Appendix II of the book, Biological Stains, has the same heading as this article. In it are published specifications for methylene blue, safranin, acid and basic fuchsin, gentian and crystal violet, pyronin, eosin Y, orange G, haematoxylin, and thionin. A few of these had already been published in articles printed in Science. The others were made public for the first time in the book just referred to.

The Commission has subsequently extended its work so that nearly 40 dyes are now sold as certified stains, as listed inside the back cover of this journal. Work has progressed far enough on quite a number of these stains so that specifications may now be drawn up for several more than those mentioned above. Accordingly, the next edition of Biological Stains will contain a considerable longer list in this appendix. So far as possible, it is planned to see that all these specifications are first published in Stain Technology; those which follow are for stains concerning which our information is now sufficiently definite to allow their formulation.

It must be understood that these specifications are not intended to furnish definite statements as to the chemistry of satisfactory stains. The object with which they were drawn up is to allow the manufacturers as much latitude in the matter of chemical composition as has been found consistent with good results in practice, and to lay the greatest stress upon the performance of the stains in actual laboratory use. The requirements listed are those which must be met by dyes submitted to the Commission for certification.

These specifications are published, moreover, with the understanding that they are subject to revision at any time. Further investigations are in progress concerning the adaptability of various dye products for different purposes, and also as to the relation of optical characters to performance in staining. The Commission reserves the right to modify the specifications for any stain as data accumulate to show the need of modification.

Specifications of Aniline Blue, Water Soluble

1. Samples of aniline blue submitted for certification must be of the type represented by the Colour Index No. 707, variously denoted as anilin blue water soluble, soluble blue, china blue, etc. This dye is a mixture of sulphonated triphenyl and diphenyl rosanilin, and no evidence is yet at hand to indicate special advantages of any particular mixture.

2. The dye content of this sample should be sufficient so that one gram will require 1.56 c.c. normal tetanous chloride solution in reduction. This should give a sample of at least 60% dye content, but the exact percentage would vary according to the relative amounts of the higher and lower homologs present.

3. The sample should prove satisfactory in the Mallory connective tissue stain (with acid fuchsin and orange G). Its performance in this technic should be tested by an histologist or pathologist familiar with the method in question.

4. The dye should act as an indicator, turning from colorless to deep blue, when tested in the absence of organic matter, between pH = 7.0 and pH = 5.0. In applying this test, an instantaneous color change is not required; but the sharper the reaction the better.

Specifications of Brilliant Cresyl Blue

1. Samples of brilliant cresyl blue submitted for certification should be of the type described under Colour Index No. 877.

2. Samples should have a dye content of over 60%, as determined by reduction of titanous chloride in an atmosphere of carbon dioxide.

3. Samples should give a satisfactory stain for platelets and reticulated erythrocytes in blood, by the technic described by Robertson in 1917 (J. Exp. Med. **26**, 221.) This test should be made by someone skilled in the technic of staining blood for this purpose.

Specifications of Congo Red

1. Congo red designed for staining purposes must be the dye denoted by Colour Index No. 370, having an absorption curve showing a maximum at about 485 mμ.

2. It should have a dye content of at least 80% as determined by reduction of titanous chloride in an atmosphere of carbon dioxide.

3. It should prove satisfactory as a counter-stain in histological work, especially in contrast to haematoxylin. In this test the stain, when applied in aqueous solution, should remain in the tissue without being washed out during alcoholic dehydration.

Specifications of Eosin B

1. Samples of eosin B should be di-nitro-di-brom fluorescein, that is, the dye designated by Colour Index No. 771, having an absorption curve showing a primary maximum at 521.5 mμ and a secondary maximum at 486 mμ.

2. The samples should have a dye content of at least 80% as determined by the color-acid precipitation method.

3. The samples should prove satisfactory in aqueous solution as a counter-stain against haematoxylin. This test should be made by a histologist skilled in the technic in question.

Specifications of Light Green, SF, Yellowish

1. Light green submitted for certification should be the dye denoted "SF, yellowish" and corresponding to Colour Index No. 670. It should have an absorption maximum at 633.5 mμ.

2. It should have a dye content of at least 70% as determined by reduction of titanous chloride in an atmosphere of carbon dioxide.

3. The sample should prove a satisfactory counter-stain in general histological work; also as a cytoplasm and cellulose stain in plant histology. It should be tested for these purposes by some one familiar with its use for the technic in question.

Specifications of Methyl Green

1. Samples of methyl green submitted for certification should be the dye corresponding to Colour Index No. 684, and having an absorption maximum at about 663.8 mμ.

2. They should have a dye content of at least 55% as determined by reduction of titanous chloride in an atmosphere of carbon dioxide.

3. The samples should give satisfactory results in plant histology as a stain for xylem and other lignified elements, and should contrast sharply with acid fuchsin when used as a counter-stain against it. It should give a sharp chromatin stain in animal histology and cytology as by the Bensley-Cowdry technic (See Lee's Microtomist's, Vademecum, 8 Ed. p. 324). This work should be tested by some one skilled in the technic in question.

Specifications of Nile Blue Sulfate

1. Nile blue sulfate submitted for certification should be of the type corresponding to the Colour Index No. 913, having an absorption curve showing a primary maximum at 644.5 mμ and a secondary maximum at 592.2 mμ.

2. It should have a dye content of 70% as determined by reduction of titanous chloride in an atmosphere of carbon dioxide.

3. When used for staining formalin fixed tissue containing fat, by the method of Smith and Mair, it should stain the free fatty acids blue and the neutralized fats red. This should be tested by the following technic:—

Formalin fixed material, sectioned by freezing method, is to be stained warm for 30 minutes with a saturated aqueous solution of the dye and differentiated in 2% acetic acid; other sections to be similarly stained using a saturated aqueous solution of the dye which has been boiled 2 hours with 0.5% sulfuric acid under a reflux condenser, and examined, mounted in glycerin, glycerin-jelly or levulose.

Specifications of Rose Bengal

1. Samples of rose bengal submitted for certification should ordinarily correspond to Colour Index No. 779, that is, should be primarily tetra-chlor tetra-iodo fluorescein. Samples of Colour

Index No. 777, that is di-chlor tetra-iodo fluorescein, will not be refused, provided they perform satisfactorily; but their nature must be correctly indicated on the labels.

2. The dye content of the samples should be at least 80%, as determined by the color acid precipitation method.

3. The dye should prove a satisfactory stain for bacteria in soil without coloring the dead organic matter, when tested by the technic of Conn (Stain Techn. 1, 128). This test should be made by some one familiar with the appearance of such a preparation when stained with a satisfactory sample.

Specifications of Phloxine

1. Samples of this dye submitted for certification should ordinarily correspond to Colour Index No. 774, that is, should be di-chlor tetra-brom fluroescein, with an absorption curve showing a maximum at about 535 mμ. Samples of the corresponding tetra-chlor compound (Colour Index 778) will be accepted and tested for the same purpose; but must be labelled "phloxine B."

2. The samples must have a dye content of 80% as determined by the color-acid precipitation method.

3. The samples must give a satisfactory cytoplasm stain when substituted for eosin in the Mallory eosin-methylene-blue technic. In this procedure the sections should be stained with a 2.5% solution of phloxine for one hour (held at 37° C) followed by 1% polychrome methylene blue (diluted 1 to 10) for one-half an hour. This test should be made by some one familiar with the technic.

Specifications of Toluidine Blue O

1. Samples of toluidine blue submitted for certification should correspond to Colour Index No. 925, having an absorption curve w th a maximum at about 635mμ.

2. Samples should have a dye content of 80%, as determined by reduction of titanous chloride in an atmosphere of carbon dioxide.

3. The samples should prove satisfactory for staining frozen sections of fresh animal tissue, and should show good metachromatic effects when app'ied in a 1% solution in 20% alcohol from 1 to 5 minutes, followed by rapid washing in water, and mounting in water. This should be judged by someone familiar with the technic.

Executive Committee,
Commission on Standardization of Biological Stains,
H. J. Conn, *Chairman.*

NOTES ON TECHNIC

DUCO, AS A MATERIAL FOR RINGING COVER GLASSES ON MICROSCOPIC SLIDES. The attention of those who prepare microscopic slides is drawn to *Duco* as a material for ringing the cover glasses on the finished slides. In a search for a desirable material for this purpose *Duco* was tried with rather satisfactory results. *Duco* is manufactured by Dupont Chemical Works, Parlin, New Jersey, U.S.A. and by Flint Paint and Varnish Limited, Toronto, Canada. One of its principal uses is as a finishing material for automobile bodies. Its desirable characteristics as a ringing material on microscopic slides are as follows:

1. It is easily obtained, being on sale in almost every town and city.
2. It is reasonable in price.
3. It comes in a variety of colors. Two colored effects may be obtained on slides if so desired.
4. It dries quickly. The whole process of placing the ring on the slide can be completed in a few minutes.
5. The finish is hard and glossy.
6. Duco may be applied directly to clean glass without previous preparation.
7. It is evidently durable.

—A. V. Mitchener, Manitoba Agr. Col.,
Winnipeg, Canada.

SOLUTIONS OF MACNEAL'S TETRACHROME STAIN. Complaints have frequently been made that altho the tetrachrome stain is as good for staining blood smears as Wright's stain, if made up fresh, the solutions do not keep well, and are entirely unsatisfactory if they must be held a few weeks before using. As the same trouble has been found in our own laboratory, the matter was referred to the National Aniline and Chemical Co. They called our attention to the directions on their label which are, apparently, sometimes overlooked or else misunderstood. These directions read as follows:

> Dissolve 0.5 gm. in 100 cc. of special pure absolute methyl alcohol by adding small portions of the powder to the alcohol warmed to 50°C. and shaking vigorously between each addition. The solution should be allowed to stand in a warm place for 24 hours and then filtered before use.

The chief difficulty in following these directions seems to be in interpreting the expression "warm place" in the last sentence. The company's representative has clarified the matter by explaining the object of this method of dissolving the stain. In the case of Wright's

stain, the reaction between the basic and acid dyes is already complete before dissolving in methyl alcohol; but in the case of the tetrachrome stain no reaction has taken place before dissolving, and its solution is therefore unstable. To obtain a stable solution the reaction must be allowed to proceed to completion, and the precipitate formed must be removed. As reactions between electrolytes take place slowly in alcohol the process must be hastened by heat. That is the reason for dissolving in alcohol at 50°C. and keeping at a warm temperature for 24 hours. If a temperature of 50° can be maintained for this length of time, the reaction takes place most satisfactorily. If placed in a 37° incubator, good results can be obtained; but it is well to allow more than 24 hours before filtering.

Following this suggestion the dye was made up in our laboratory as directed and kept at about 50° for rather more than 24 hours before filtering. The solution has subsequently been used at two different times, once a full month after its preparation. The blood stain obtained is entirely satisfactory. There is, in fact, no comparison between the results thus obtained and those secured when the stain was dissolved similarly but with the words "warm place" interpreted to mean a warm spot in an ordinary room.

Attention is called to this matter, thinking that others may have made the same mistake.—Editor.